BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

REMARKS

Claim 11 is pending in this application.

A substitute Abstract of the Disclosure is enclosed. The Examiner objected to the prior version because the number words therein exceeded the number of words permitted.

A substitute Specification is enclosed. The Examiner objected to the prior version for various informalities. Significantly, the Examiner objected to the specification because several sequences in the specification were not in the Sequence Listing for this application. In compliance with 37 CFR 1.821 - 1.825, the substitute specification identifies all sequences with a SEQ. ID. NO.

In light of the substitute specification, a new Sequence Listing for this application is enclosed herewith. The CRF copy and the paper copy are identical. No new matter has been added.

Claim 11 has been rejected under 35 U.S.C. 101. Applicants respectfully submit that, in light of the foregoing amendments to claim 11, the §101 rejection of claim 11 is now moot and should be withdrawn.

Claim 11 stands rejected under 35 U.S.C. 112. In particular, claim 11 is rejected because it is not apparent from the specification or claims that the hybridoma producing MAb 448/30/7 is deposited at a recognized depository. As stated in the enclosed Declaration, On or before August 26, 2003, the hybridoma producing MAb 448/30/7 was deposited with BCCM™/LMBP, Department of Molecular Biology, Ghent University, Fiers-Schell-Van Montagu Building,Technologiepark 927,B-9052 Zwijnaarde, Belgium, and assigned Deposit Number LMBP 6047CB. The deposit was made under the provisions of the Budapest Treaty. Access to the deposit will be available during pendency of the patent application making reference to the deposit to one determined by the Commissioner to be entitled thereto under § 1.14 and 35 U.S.C. 122. All restriction upon public access to the deposit will be irrevocably removed upon the grant of a patent on this application. The deposit will be replaced if viable samples cannot be dispensed by the depository as required.

The Examiner asserts that claim 11 is vague in the recitation of P64K antigen of *Neisseria meningitides* B:4:P1.15. The Examiner questions the particular nature of the P64K antigen. In response, the Applicants respectfully submit that the P64K antigen is well known to those skilled in the art. In particular, P64K is a high molecular mass protein that is common to many meningococcal isolates. Further details regarding P64K are provided in the enclosed article, "B-cell epitope mapping of the *Neisseria meningitides* P64k protein using overlapping peptides," Biotechnol. Appl. Biochem. (2000) 32, pp. 1-8.

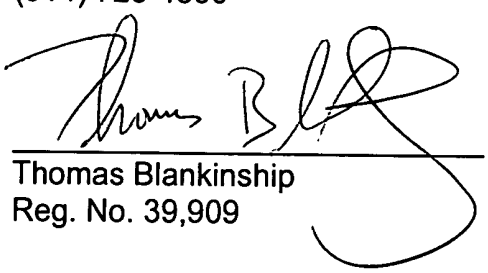
It light of the foregoing, the Applicants respectfully submit that the §112 rejections of claim 11 are now moot and should be withdrawn.

Claim 11 has been rejected under 35 U.S.C. 102(b) as being anticipated by Nazabul et al. Also, claim 11 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Rodriguez et al. or Niebla et al.

Applicants respectfully submit that none of the cited references, either alone or in any combination, disclose or even suggest an isolated monoclonal antibody 448/30/7 that is produced from a hybridoma deposited with BCCM™/LMBP, Department of Molecular Biology, Ghent University, Fiers-Schell-Van Montagu Building,Technologiepark 927,B-9052 Zwijnaarde, Belgium, under deposit number LMBP 6047CB, and that is specific for the stabilizer peptide produced from the first 47 amino acids of the N-terminal end of the P64K antigen of *Neisseria meningitides* B:4:P1.15. Therefore, the Applicants respectfully disagree with the Examiner's rejections of claim 11, and submit that the rejections of claim 11 should be reconsidered and withdrawn.

Date: September 26, 2003

Respectfully submitted,
LACKENBACH SIEGEL, LLP
One Chase Road
Scarsdale, NY 10583
(914) 723-4300



Thomas Blankinship
Reg. No. 39,909

MARKED UP VERSION OF THE SPECIFICATION

EXPRESSION SYSTEM OF HETEROLOGOUS ANTIGENS AS FUSION PROTEINS

The instant application is a Divisional application of SN 08/930,917, which was filed as a national stage 371 application of the PCT application, PCT/CU97/00001, filed 01/17/1997, with a priority claim to application 10/96, filed 01/17/1996 in Cuba.

1

TECHNICAL SECTOR

The present invention is related to the field of the Biotechnology and the genetic engineering, particularly to the expression of heterologous proteins in microbial hosts through their fusion to bacteria peptides, using the technology of the recombinant DNA.

PREVIOUS ART

The usefulness of the technology of the recombinant DNA to produce proteins of any origin in *E. coli* has been extensively demonstrated. For this, an important amount of vectors have been developed, although new variants are necessary due to the fact that, frequently each gene to clone and to express represents an individual case (Denhardt, D. T. and Colasanti, J.; Vectors ed., Butterworths, Stoneham, MA, Biotechnology 10, 179-203, 1988 ~~M, pp.1791987~~ and Lukacsovich, T. *et al.*, Journal of Biotechnology, 13, 243-250, 1990 ~~2431990~~).

MARKED UP VERSION OF THE SPECIFICATION

The intracellular synthesis has been the most used strategy for the obtainment of heterologous polypeptides in *E. coli*, due to the high expression levels reachable (Goeddel, D. V, Methods Enzymol., 185, 3-7, 1990). However, factors such as the sensitivity to proteases of the host or toxicity of the expressed protein can reduce significantly said levels, independently of the use of regulatory sequences of high efficiency (Lee ~~Reads~~, C. A. and Saier, M. H., J. Bacteriol., 153, 685-692, 1983; Gwyn, G. W., Membrane Protein Expression Systems: A ~~To~~ User's Guide, Portland Press, London, UK, 29-82, 1992). The cloning of nucleotide sequences encoding for proteins of interest in suitable vectors, in frame with sequences of nucleic acid that encode stable polypeptides in the host cell, gives rise to the expression of hybrid products in the cytoplasm, known as fusion proteins (Marston, F. A. O., Biochem. J. 240, 1-12, 1986 ~~11986~~). Such polypeptides are generally less sensitive to proteolytic degradation by the host or less toxic due to the formation of inclusion bodies, which results in higher expression levels to those obtained without the use of the stabilizer peptide (Itakura, K. et al., Science, 198, 1056-1063, 1977 ~~10561977~~). In addition, this kind of expression facilitates and cheapens the initial steps of the purification if different methods for the subsequent renaturation of the recombinant product are available (Fischer, B., Sumner, I. and Goodenough, P., Biotechnol. Bioeng., 41, 3-13, 1993).

The inclusion bodies are insoluble protein aggregates that appear as electrodense bodies in the cytosol ~~citosol~~ during the expression of many recombinant proteins in *E. coli* (Rinas, U. ~~Or.~~ and Bailey, J., Appl. Microbiol. Biotechnol., 37, 609-614, 1992). They are the result of the interaction between polypeptides partially folded, whose

aggregation is thermodynamically favored due to the exposition, within them, of hydrophobic residues to the solvent (Kiefhaber, T., Rudolph, R. et al., Biotechnology, 9, 825-829, 1991). The slow folding in the bacterial cytosol ~~citosol~~ of many eukaryotic proteins, due to the abundance of disulfide bridges-forming amino acids (Cysteino) or beta-turn-forming amino acids (Proline) has stimulated the abundant use of them as stabilizer peptides. Examples of the former are the use, with this purpose, of polypeptides with binding activity to antibodies, coming from the globulin of the fat of the human milk (HMFG), according to the international patent application PCT No. WO 9207939 A2 920514; from constant regions of the immunoglobulins, as described in the European patent application No. EP 0464533 A1 920108; from the human angiogenin (European patent application No. EP 0423641 A2 910424), of the growth hormone (EP 0429586 A1 910605), the glutatione-S-tranferase (WO 8809372 A1 881201) and of the swine adenylate quinase (EP 0423641 A2 910424 and EP 0412526 A2 910213).

However, the use of stabilizer polypeptides that constitute a significant part of the fusion protein has some disadvantages if the former is a vaccine candidate, since the presence of the foreign sequences can alter the natural order of the B and T cell epitopes (Denton, G., Hudecz, F., Kajtar, J. et al., Peptide Research, 7, 258-264, 1994) or the processing of the same by the antigen presenting cells (Del Val, M., Schlicht, H., Ruppert, T., et al, Cell, 66, 1145-1153, 1991), being able to even affect seriously the immunogenicity of the candidate by the phenomenon of specific-epitope suppression (Etlinger, H., Immunol. Today, 13, 52-55, 1992).

MARKED UP VERSION OF THE SPECIFICATION

As a result of the aforementioned phenomenon, in some cases, small fragments that still stabilize the expression have been tried to be defined. For example, the German patent application No. 35 41 856 A1 (Hoechst AG) reports the possibility of using a stabilizer peptide conformed by at least the first 95 amino acids of the N-terminus of the human protein Interleukine (IL-2) to obtain fusion proteins in an insoluble form synthesized in *E. coli*. Similarly in the European Patent Applications No. 0 416 673 A2 and No. 229 998 from the same company, a stabilizer peptide consistent in the first 58 or 38 amino acids of said protein, is used. In the European patent No. 416 673 B1, the first 58 amino acids of the IL-2 are also used, and a similar strategy is followed, with this purpose, in the case of use of N-terminal fragments of the human seroalbumin (European patent application No. EP 0423641 A1 920212); the activator peptide III of the connective tissue (WO 90136647 A1 901115) and fragments of the human kallikrein (EP 0381433 A1 900808). These inventions give solution to the previous problem, but the fusion polypeptides obtained can not be included in vaccine preparations for use in humans, due to the possibility of induction of autoimmune diseases for the presence in them of homologous or identical sequences to human proteins.

The alternative of using stabilizer polypeptides of bacterial origin--and therefore, without cross reactivity with antigens of human origin--for intracellular expression, has also been explored with success. One of the most used proteins with this end has been the β-galactosidase of *E. coli* (Itakura, K. et al., Science, 198, 1056-1063, 1977 ~~10561977~~) or portions of it (German patent application No. EP 0235754 A2 870909, of the company Hoechst AG). The principal disadvantage of this system is the great size of

MARKED UP VERSION OF THE SPECIFICATION

this protein which provokes that the desired peptide only represents a small portion of the total hybrid protein (Flores ~~Flowers~~, N. et al., Appl. Microbiol. Biotechnol. 25, 267-271, 1986 ~~2671986~~; Goeddel, D. V. et al., P.N.A.S. USA, 76, 106-110, 1979). Similar problems are presented with the use of the C fragment of the tetanus toxoid and the exotoxin of *Pseudomonas* sp. (International Patent Application PCT WO 9403615 A1 940217 and European Patent Application EP 0369316 A2 900523). An expression variant that is very promising is the use of fusions with the thioredoxin of *E. coli* (PCT Patent application No. WO 9402502 A1 940203), that uses the property of being liberated from the cell by osmotic stress (el Yasgoubi ~~Elyaagoubi~~, A., Kohiyama, M., Richarme, G., J. Bacteriol., 176, 7074-7078, 1994) to facilitate the purification. However, this outline is not functional for the obtainment of inclusion bodies, since the same are not freed through this procedure.

Many of these problems have been solved with the design of modular fusion proteins. In these, the stabilizer peptide is separated from the protein of interest by a spacer that permits the independent folding of both, and whose amino acid sequence makes it susceptible to the attack of specific endopeptidases. If there is a ligand that recognizes the chosen stabilizer, it is possible to purify the fusion polypeptide by affinity chromatography and finally separate it from the stabilizer through the treatment with different proteases (Cress, D., Shultz, J. and Breitlow, S., Promega Notes ~~you Note~~, 42, 2-7, 1993). An additional advantage is the possibility of exploiting this molecular interaction for the follow-up of intermediate steps of the purification, without the need of antibodies for each protein to express. A well-known example of that is the use of the

affinity of histidine (Hys) with some metals like nickel (Ni) and zinc (Zn) in systems composed of a stabilizer with 6 His in tandem and an affinity matrix of nickel chelates, according to what is described in the PCT Patent application No. WO 9115589 A1 911017 of The Upjohn Co. In spite of all this, this kind of expression system does not function in all the cases, since, among other reasons, the protein of interest can have restriction sites for the chosen protease, or be folded so that the spacer is available to the solvent (Uhlen, M. and Moks, T., Meth. Enzymol. 185, 129-143, 1990; Cress, D., Shultz, J. and Breitlow, S., Promega Notes ~~you Note~~, 42, 2-7, 1993), to interfere with the binding between the stabilizer and the affinity matrix (New England Biolabs, The NEB Transcript, 3, 1, 1991 ~~14~~), or simply to require, for its purification, conditions that affect its biological activity. For these reasons it is desirable to have different variants, since each protein to express can represent a particular case. With this purpose, there have been developed stabilizer peptides based on the maltose binding protein of *E. coli* (MalE), which have affinity for the amylose resins (European Patent Application EP 0426787 A1 910515); in the chloramphenicol acetyl transferase enzymes (European Patent Application No. EP 0131363 A1 850116) or in the glutatione-S-transferase (European Patent Application No. EP 0293249 A1 88130, of the Amrad Corp., Ltd.) obtainable with matrixes of immobilized substrate; in the protein A of *Staphylococcus aureus*, according to the patent application PCT WO 9109946 A1 910711; and in the 12.5 kDa subunit ~~subunity~~ of the transcarboxylase complex of *Proprionibacterium shermanii*, which is biotinylated *in vivo* and permits the purification based on the affinity of the biotin to avidin (Cress, D., Shultz, J. and Breitlow, S., Promega Notes, 42, 2-7, 1993; patent applications No. EP 0472658 A1 920304 or WO 9014431 A1 901129).

MARKED UP VERSION OF THE SPECIFICATION

Of particular interest is the method described in the European Patent Application EP 0472658 A1 920304 or WO 9014431 A1 901129, developed by Biotechnology Research and Development Cor*pora*tion, along with the University of Illinois, USA. In this application an expression system is described that uses the lipoic acid binding domain of the dihydrolipoamida acetyltransferese (EC 2.3.1.12), also known as the E2 subunit of the pyrovate dehydrogenase complex of *E. coli.* This domain is modified postranslationally *in vivo* by the addition of a lipoic acid molecule to the nitrogen of one of its lysines (Guest, J. R., Angier, J. S. and Russell, G. C., Ann. N.Y. Acad. Sci., 573, 76-99, 1989), which is exploited for the purification and identification of fused proteins through the use of an antibody that recognizes only lipoylate domains.

This method, however, has a number of drawbacks. First of all, it is known that the over expression of proteins containing binding domains to the lipoic acid exceeds the capacity of cellular lipoylation, producing as a consequence no lipoylates domains (Miles ~~Thousands~~, J. S. and Guest, J. R., Biochem. J., 245, 869-874, 1987; Ali, S.T. and Guest, J.R., Biochem. J., 271, 139-145) or octanoilates (Ali, S.T., Moir, A.J., Ashton, P.R. et al. Mol. Microbiol., 4, 943-950, 1990; Dardel, F., Packman, L.C. and Perham, R.N., FEBS Lett. 264, 206-210, 1990 ~~295,13-16~~), which can reduce the yield during purification by immunoaffinity. In second place, there are a group of diseases of a supposed autoimmune origin which have as a common factor the presence of antibodies that recognize specifically the lipoic acid in the context of these domains. Among them are primary biliary cirrhosis, a chronic disease characterized by the

inflammation and progressive obstruction of the intrahepatic bile ducts (Tuaillon, N., Andre, C., Briand, J.P. et al., J. Immunol., 148, 445-450, 1992); and hepatitis and the hepatitis provoked by halothane, an anesthetic of wide use that derivatizes some proteins by the formation of trifluoroacetyl lysine (Gut, J., Christen, U. ~~Or.~~, Frey, N. et al, Toxicology, 97, 199-224, 1995). The serum of the patients with this disease recognizes said complexes, whose molecular structure is mimicked by the lipoic acid in the context of the dihydrolipoamide acetyl transferases (Gut, J., Christen, U. ~~Or.~~, Frey, N. et al., Toxicology, 97, 199-224, 1995). For this reason it is desirable to avoid the presence of the lipoic acid in such peptides if the fusion proteins that contain it constitute vaccine candidates for use in humans.

DISCLOSURE OF THE INVENTION

An object of the present invention is a procedure for the expression to high levels of heterologous proteins as fusion polypeptides in *E. coli*, which is based on the use of a stabilizer sequence derivative from the first 47 amino acids of the P64K antigen of *N. meningitidis* B:4:P1.15 (European Patent application No. 0 474 313 A2) that confers on them the capacity of being expressed as inclusion bodies. Said sequence, though presents homology with part of the lipoic acid binding domain of the dihydrolipoamide acetyl transferases, has been genetically manipulated to eliminate the possibility of modification for itself and presents the advantage of being lowly immunogenic. This procedure also includes the use of a monoclonal antibody that specifically recognizes

the mentioned stabilizer, permitting the immunodetection of any protein fused to the same.

Particularly, in the present invention, a recombinant plasmid as an expression vector is used which carries said sequence under the control of the tryptophan promoter (ptrip) of *E. coli*, followed by restriction sites XbaI, EcoRV and BamHI. These permit the in frame cloning of DNA fragments encoding for polypeptides of interest. This vector also includes a terminator of the transcription of the gene 32 of bacteriophage T4 and a resistance gene to ampicillin as selection marker.

This procedure makes possible also the inclusion of the fusion polypeptide obtained in vaccine preparations destined to be used in humans; and the nature of the stabilizer peptide employed permits the generation of protective immune response against the foreign protein or the multiepitopic peptide bound to it.

A novelty of the present invention is the genetic manipulation and the use of an homologous stabilizer peptide to part of the lipoic acid binding domain of the dihydrolipoamide acetyl transferases, for the production of fusion proteins by recombinant DNA technology in *E. coli*. Particularly, novelties of the present invention are the use, with the previous objective, of a stabilizer peptide derivative of the first 47 amino acids of the P64K antigen of *N. meningitidis* B:4:P1.15 (European Patent application No. 0 474 313 A2), and a monoclonal antibody that specifically recognizes the stabilizer.

MARKED UP VERSION OF THE SPECIFICATION

The values obtained (FIG. 12) show that the titers against the V3 regions are similar between the varying IL2-22+MEP (TAB4) and P64K-47+MEP (TAB9). Though the recognition frequency of the peptides is slightly greater for the TAB9, this difference is not meaningful statistically ($p<0.05$). In conclusion, the immunogenicity of the heterologous protein is affected by the stabilizer P64K-47 in a minimal way, and comparable to other expression systems currently in use.

DESCRIPTION OF THE FIGURES

FIG. 1: Nucleotide sequence (SEQ. ID. NO. 1) of the gene *lpdA* gene coding for P64K. It is shown in *italic* the sequence added in the plasmid pM-6 (European Patent application No. 0 474 313 A2), absent originally in the gene *lpdA*.

FIG. 2: Reactivity of the polyclonal serum of mouse against peptides of the P64K. A minimal value of 0.4 optical density units to consider the result as positive was chosen.

FIG. 3: Amino acid sequence (SEQ. ID. NO. 2) of the stabilizer, deduced of the DNA sequence (SEQ. ID. NO. 3) amplified by PCR from plasmid pM-6. The underlined sequences correspond to the oligonucleotide primers.

FIG. 4: Strategy for the construction of plasmid pM-83.

MARKED UP VERSION OF THE SPECIFICATION

FIG. 5: Results of the search of homology between the sequences of the stabilizer ('Query') (SEQ. ID. NO. 4) and those present in the SWISS-PROT ('Sbjct') (SEQ. ID. NO. 5) base, using the BLASTP program. The corresponding income for human proteins or for mammals proteins are only shown. P(N) represents the probability of finding N equal alignments within a base composed of random sequences; the significance of the homology diminishes with the value of P(N). Identical residues are represented with their codes of one letter; the conservatives substitutions with a '+', and the differences are not indicated.

FIG. 6: Results of the search of homology between the sequences of the stabilizer ('Query') (SEQ. ID. NO. 6) and all the possible translations of the sequences of the EMBL Data Library ('Sbjct') (SEQ. ID. NO. 7), using the program TBLASTN. The corresponding income to human proteins or mammal proteins are only shown. P(N) represents the probability of finding N equal alignments within a base composed of random sequences; the significance of the homology diminishes with the value of P(N). Identical residues are represented with their code of one letter; the conservative substitutions with a '+', and the differences are not indicated.

FIG. 7: Strategy for the construction of plasmids pTAB4 and pTAB9.

FIG. 8: Amino acid (SEQ. ID. NO. 8) and nucleotide (SEQ. ID. NO. 9) sequences of the MEP TAB9.

MARKED UP VERSION OF THE SPECIFICATION

FIG. 9A: General structure of the MEP TAB4 and TAB9.

FIG. 9B: General structure of the MEP TAB13.

10A: Comparison of the expression of the genes *porA*, *opc* and the MEP under stabilizer derivatives from the human IL-2 or from the first 47 amino acids of the P64K antigen. hIL2-58 refers to the first 58 amino acids of the human IL-2, hIL2-22 to the first 22, and P64K-47 to stabilizer derivative from the first 47 amino acids of the P64K antigen.

10B: Comparative analysis by SDS-PAGE of the expression of the MEP in the plasmids TAB4 and TAB9. Lane A: Molecular weight markers; B: Total proteins of the strain W3110 *trpA905*; C: Total proteins of W3110 *trpA905*+pTAB4; D: Purified TAB4; E: Total proteins of W3110 *trpA905* pTAB9; F: Purified TAB9.

10C: Expression of TAB9 in inclusion bodies. A: Soluble proteins of the sample. B: Insoluble proteins or of membrane.

FIG. 11: Western blotting using MAb 448/30/7 with total protein samples of *E. coli* MM294 transformed with: 1: Negative control, 2: pM-6 (P64K), 3: pM-82 (P64K-47+*porA*), 4: pTAB13 (P64K-47+MEP), 5: pFP15 (IL-2), 6: pM-134 (P64K-120), 7: pILM-28 (IL2-58+*porA*). The molecular weight markers are indicated on the left.

MARKED UP VERSION OF THE SPECIFICATION

FIG. 12: Reciprocal of the titer value by ELISA of the rabbits immunized with TAB4 and TAB9. GM: Geometric mean of the reciprocal of the titers anti V3; R: Percent of reactivity with the V3 peptides.

EXAMPLES

Example 1

The LpdA antigen of *N. meningitidis* (P64K, LpdA) is a protein of 594 amino acids that belongs to the family of the dihydrolipoamide dehydrogenases (EC 1.8.1.4) and specifically, to a new subgroup within them, characterized by possessing a lipoic acid binding domain, analogous to the one present in the dihydrolipoamide acetyltransferases, in its N-terminal portion (Kruger, N., Oppermann, F. B., Lorenzl, H. and Steinbuchel, A., J. Bacteriol., 176, 3614-3630,1994; Hein, S. and Steinbuchel, A., J. Bacteriol., 176, 4394-4408, 1994). The LpdA protein has been cloned and over expressed in *E. coli*, with the addition of 5 amino acids (MLDKR) in its N-terminal end (European Patent application No. 0 474 313 A2; FIG. 1; SEQ. ID. NO. 1). Although the denominations LpdA and P64K are equivalent, the name P64K for referring to the recombinant protein will be used.

In order to determine the immunogenicity of different fragments from said antigen and to analyze the possibility of using the less immunogenic as stabilizer peptide, the epitopes

MARKED UP VERSION OF THE SPECIFICATION

for B cells present in P64K were located through the evaluation of the reactivity of a polyclonal serum anti-P64k against synthetic peptides.

With this aim, the P64K protein was purified (European Patent application No. 0 474 313 A2) through hydrophobicity ~~hybrofobicity~~ chromatography of in Butyl-TSK and gel-filtration; and it was denatured by precipitation with trichloroacetic ~~trichloroacetic~~ acid (TCA) neutralizing them with NaOH and balancing in phosphate buffer by gel-filtration chromatography. This preparation was used to immunize 30 mice Balb/c by subcutaneous route with doses of 20 µg adjuvated ~~adyuvated~~ to 2 µg of aluminum hydroxide (day 0), which were then boosted with the same antigen 7 and 21 days later. Sera were collected 28 days after the first extraction. The sera obtained were combined, and the resulting mixture was aliquoted ~~alicuoted~~ and stored at -20° C.

Furthermore, 59 peptides of 20 amino acids (a.a.) each covering the entire sequence of the recombinant protein and overlapped by 10 a.a., were synthesized using a commercial kit for the synthesis in solid phase (Multipin Peptide Synthesis System, Chairon Mimotope Pty., Ltd., USA) in 96 wells--plates format and following the instructions given by the manufacturer. These were subsequently numbered from the N-terminal end of the protein. The reactivity of the serum antiP64k against these peptides was determined using a dilution 1:2000 of the same, and the format of immunoassay used was the same as one recommended by the manufacturer of the previous commercial kit.

MARKED UP VERSION OF THE SPECIFICATION

The results are shown in the FIG. 2, in which absorbance values for each peptide are represented. It is evident that the first 110 amino acids (represented by the peptides 1 to 11) form a poorly immunogenic segment in spite of the denaturation of the immunogen, which can even expose cryptic epitopes. This segment includes essentially the lipoic acid binding domain and the spacer region rich in Proline and Alanine that link it to the rest of the protein. This result demonstrates that the stabilizer peptide (or derivative fragments from it) can be used advantageously as stabilizer peptides, due to the small influence that it would have on the immunogenicity of the polypeptides to which it is fused. This advantage is especially important if the fusion polypeptide constitutes a vaccine candidate.

Example 2

In order to express different heterologous proteins in *E. coli* through their fusion to the lipoic acid binding domain of the P64K antigen of *N. meningitidis* B:4:P1.15, the expression vector pM-83 was constructed, in which the sequence coding for a stabilizer peptide, derived from the first 47 amino acid of said protein was introduced (SEQUENCE IDENTIFICATION NUMBER: 10 ~~1~~). This sequence is cloned under the control of the tryptophan promoter of E. coil, including the terminator of the bacteriophage T4 as signal for the transcription termination, and the ampicillin resistance gene as the selection marker.

MARKED UP VERSION OF THE SPECIFICATION

To obtain the PM-83 expression vector, the stabilizer peptide was first amplified using the Polymerase Chain Reaction (PCR) (Randall, K. et al., Science, 42394, 487-491, 1988) from the plasmid pM-6, which carries the nucleotide sequence coding for the P64K antigen (European Patent application No. 0 474 313 A2, FIG. 1, SEQ. ID. NO. 1). For this purpose, the oligonucleotide primers 1573 (SEQ. ID. NO. 11) and 1575 (SEQ. ID. NO. 12) were used, which introduce NcoI and XbaI restriction sites in the amplified DNA fragment that correspond with the amino and carboxyl terminal ends of the stabilizer encoded by it:

NcoI

1573: 5' TTCCATGGTAGATAAAAG 3'(SEQUENCE IDENTIFICATION NUMBER: 11 ~~2~~)

XbaI

1575: 5' TTTCTAGATCCAAAGTAA 3' (SEQUENCE IDENTIFICATION NUMBER: 12 ~~3~~)

The amino acid sequence encoded by the resultant stabilizer is shown in FIG. 3 (SEQUENCE IDENTIFICATION NUMBER: 15 ~~6~~). The introduction of the restriction site NcoI changes Leucine 2 for Valine; and the primer 1575 eliminates the sequence ETD (position 45-47), introducing in its place the sequence DLE. In this way the binding Lysine of the lipoic acid (position 48) does not form part of the stabilizer, and the vicinity of it, which is highly conserved in these domains (Russell, G. C., Guest, J. R., Biochim. Biophys. Record, 1076, 225-232, 1991) is altered. All this guarantees the elimination of the possibilities of posttranslational lipoylation of the fusion proteins that contain these

domains, and the generation, during the immunization with these proteins, of auto antibodies of similar specificity to those present in the patients of primary biliary cirrhosis (Tuaillon, N., Andre, C., Briand, J. P. et al., J. Immunol., 148, 445-450, 1992).

Plasmid pM-83 was constructed through the cloning of this fragment (SEQUENCE IDENTIFICATION NUMBER: 14 ~~5~~) previously digested XbaI/NcoI in the plasmid pILM-29 (Guillen, G., Loyal, M., Alvarez, A. et al., Acta Biotecnologica, 15, 97-106, 1995). The pILM29 plasmid contains the gene for the protein Opc (5c) of *N. meningitidis* fused to a stabilizer peptide consistent in the first 58 amino acids with human IL-2, so that such cloning removes the fragment of IL-2 and fuses the Opc to the stabilizer of the P64K protein (FIG. 4). From the resultant plasmid, designated pM-80, the *opc* gene was excised using the enzymes XbaI and BamHI, and in its place was cloned an adapter formed by the hybridization of the oligonucleotides 1576 (SEQ. ID. NO. 16) and 1577 (SEQ. ID. NO. 17), which introduce restriction sites XbaI, EcoRV and BamHI in the extreme 3' of the stabilizer fragment:

1576 5' CTAGATTTGATATCAG 3' (SEQUENCE IDENTIFICATION NUMBER: 16 ~~7~~)

1577 3' TAAACTATAGTCCTAG 5' (SEQUENCE IDENTIFICATION NUMBER: 17 ~~8~~)

This plasmid was designated pM-83 (FIG. 4). The insertion of all the DNA fragments and oligonucleotides, as well as the maintenance of the correct reading frame, were verified by DNA sequencing according to Sanger, F. et al., (PNAS, USA, 74: 5463-5467, 1977).

MARKED UP VERSION OF THE SPECIFICATION

Example 3

It is important that the stabilizer does not contain regions of high homology with human proteins if the resulting fusion protein is a vaccine candidate. The determination of the similarity of the stabilizer peptide of the pM-83 (EXAMPLE 2) with human proteins was accomplished through a search of homology in the data bases EMBL Data Library v.38 (Curl, C. M., Fuchs, R., Higgins, D. G. et al., Nucl. Acids Beast. 21, 2967-2971, 1993) of nucleotide sequences, and SWISS-PROT v.38 (Bairoch, A. and Boeckmann, B., Nucl. Acids Beast 21, 3093-3096, 1993) of amino acid sequences; both March 1994 versions. For this search two of the programs BLAST were used (Altschul, S. F., Gish, W., Miller, W., Myers, And. W. and Lipman, D. J., J. Mol. Biol., 215:403-410, 1990): BLASTP, that compares one amino acid sequence against a base of protein sequences (in this case SWISS-PROT) and TBLASTN, that compares an amino acid sequence against all the translations in both directions and in all the reading frames of a base of nucleotide sequences, as in this case the EMBL Data Library; in both cases it was used a valorization matrix PAM120 [Dayhoff, M. O., Schwartz, R. M. and Orcutt, B. B., in: Dayhoff, M. Or. (of.), Atlas of Protein Sequence and Structure, 5, supl.3, 345-352, Natn. Biomed. Beast. Found., Washington, 1978].

The result can be observed in FIGS. 5 and 6, in which the sheets of the respective results of the BLASTP and the TBLASTN are shown (homologous sequences of prokaryotes or inferior eukaryotes have been omitted for a better understanding). It is

obvious that no human protein or proteins from any other mammal presents meaningful similarities with the stabilizer derived from the P64K; since the homologies detected by both algorithms (in the human and rat pyruvate kinases; and the C-terminal end of the human and canine mucines) present a highest casual occurrence probability (as a comparison point, the same probability, for the case of the dihydrolipoamide acetyltransferase of *Azotobacter vinelandii*, it is 3.7×10^{-5}).

Of all of the above mentioned it can be concluded that the use of said stabilizer in vaccine candidates is absolutely sure.

Example 4

The capacity of the present stabilizer in the pM-83 of permitting the intracellular synthesis at high levels and in the form of inclusion bodies was evaluated, comparing the expression of several proteins fused to the first 22 or 58 amino acids of the human Interleukin-2 (IL-2), a fusion peptide often used with this end, or fused to the first 47 a.a. of the P64K antigen modified according to is described in the EXAMPLE 2.

For this purpose the genes coding for the outer membrane proteins of *N. meningitidis* B:4:P1.15 *PorA* and Opc were cloned into the vectors pFP15 (hIL2-58; European Patent No. 416 673 B1) or pM-83 (P64K-47) ; and in the vectors pISL31 (hIL2-22, Castellanos-Sierra, L. R., Hardy, E., Ubieta, R., et al., paper submitted) or pM-83, the genes coding for a multiepitopic polypeptide (MEP) that includes immunogenic regions of several

isolates of the Human Immunodeficiency Virus, HIV. The resultant expression plasmids are: pILM-28 (IL2-58 *PorA*; Guillen, G., Alvarez, A., Lion, L., et al., 494-498 in: Conde-Gonzalez, C. J., Morse, S., Rice, P. et al. (eds) ., Pathobiology and Immunobiology of Neisseriaceae, Instituto de Salud Publica Nacional, Cuernavaca, Mexico, 1994), pM-82 (P64K-47 *PorA*; Niebla, O., Alvarez, A., Gonzalez, S. et al., 85-86 in: Evans, J. S., Yost, S. and Maiden, M. C. J. et al. (eds.)., Neisseria 94: Proceedings of the IX International Pathogenic Neisseria Conference, Winchester, England, 1994), pILM-29 (IL2-58 Opc; Guillen, G., Leal, M., Alvarez, A. et al., Acta Biotecnologica, 15, 97-106, 1995), pM-80 (EXAMPLE 2, FIG. 4), pTAB4 (IL2-22+MEP) and pTAB9 (P64K-47 MEP).

The TAB4 and TAB9 proteins are multiepitopic polypeptides (MEP) that include several copies of the central part of the variable region 3 (V3) of the gp120 protein of the HIV-1. For the construction of these MEP, 15 central amino acids of the region V3 of the following isolates were selected:

LR150: SRGIRIGPGRAILAT (SEQUENCE IDENTIFICATION NUMBER: 18 ~~9~~)

JY1: RQSTPIGLGQALYTT(SEQUENCE IDENTIFICATION NUMBER: 19 ~~10~~)

RF: RKSITKGPGRVIYAT(SEQUENCE IDENTIFICATION NUMBER: 20 ~~11~~)

MN: RKRIHIGPGRAFYTT(SEQUENCE IDENTIFICATION NUMBER: 21 ~~12~~)

BRVA: RKRITMGPGRVYYTT(SEQUENCE IDENTIFICATION NUMBER: 22 ~~13~~)

IIIB: SIRIQRGPGRAFVTI(SEQUENCE IDENTIFICATION NUMBER: 23 ~~14~~)

MARKED UP VERSION OF THE SPECIFICATION

These regions are bound by a spacer peptide of five amino acids, of sequence AGGGA (SEQUENCE IDENTIFICATION NUMBER: 26 ~~17~~). To achieve this, the DNA sequence coding for the V3 epitopes bound by the spacer peptide was obtained by chemical synthesis (SEQUENCE IDENTIFICATION NUMBER: 30 ~~21~~) and was cloned under the control of the tryptophan promoter, fused to the first 22 amino acids of the human IL-2 (FIG. 7). From the resultant plasmid, designated pTAB3, a fragment containing the gene for the MEP, the tryptophan promoter and the T4 terminator was excised by digestion with the enzymes ScaI and HindIII, and is cloned into pUC19 (Yanisch-Perron, C. et al., 1985, Gene 33, 103-119) to obtain the pTAB4 (FIG. 7). Finally, the pTAB9 was constructed eliminating the sequence coding for the stabilizer derived from the human IL-2 by digestion with the enzymes NcoI and XbaI, and cloning, in its place, a fragment coding for the first 47 amino acids of the P64K antigen obtained by polymerase chain reaction (PCR), as is described in the EXAMPLE 2. The sequence of the resultant MEP (SEQ. ID. NO. 8) is shown in FIG. 8, and its organization in FIG. 9A.

The host strains of *E. coli* K-12 used for all these plasmids were the W3110 (Hill, C. W., and Hamish, B, W. Proc. Natl. Acad. Sci., 78, 7069, 1981; Jensen, K. F., J. Bacteriol., 175, 3401-3407, 1993) for pILM-28, pILM-29, pM-80 and pM-82; and the W3110 *trpA905*, for pTAB4 and pTAB9. The expression was achieved in all the cases by inoculating a culture of 5 mL of LB medium (Sambrook, J., Fritsch, Y. F. and Maniatis, T., Molecular Cloning: To Manual Laboratory, Cold Spring Harbor Laboratory Press, 1989, New York, USA) with ampicillin (Ap) to 50 µg/mL and tryptophan (W) to 100 µg/mL, which was grown 12 h at 37° C. Said culture was used to inoculate a culture of

MARKED UP VERSION OF THE SPECIFICATION

50 mL of LB-Ap (pTAB4 and pTAB9) or a defined medium compound by M9 salts (Miller, J. H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, 1972, New York, USA), glucose to 1%, casein hydrolyzate to 1%, $CaCl_2$ 0.1 mM, $MgCl_2$ 1 mM and Ap to 50 ug/mL (plLM-28, plLM-29, pM-80, pM-82), those which were grown 12 h to 37° C. and 250 r.p.m. After this time, total protein samples were taken and analyzed by denatured polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli, O. K., Nature, 277, 680, 1970s) and staining with Coomassie Brilliant Blue R-250. The expression percent was analyzed in a densiometre of laser Bromma-LKB. Their cellular location was determined by lysing the cells through treatment combined with lysozyme and ultrasound, after something then the soluble proteins were separated from the insoluble ones by centrifugation. The insolubility of the protein was used as criterion to assume its expression as inclusion bodies, since other conditions under which they can exhibit said behavior (association to membranes or to the peptide glycan) are unlikely in this case.

A summary of the results can be seen in the FIG. 10A. In all the cases the expression under the stabilizer derived from the P64K is comparable to the expression obtained when fused to peptides of the IL-2 concerning the relationship of heterologous protein: total cellular protein (see FIG. 10 B for the case of the MEP), which confirms the capacity of the pM-83 to be used as vector for the expression of fusion peptides. It is worth noting that these polypeptides are too hard to express in *E. coli* if they are not fused, either by their small size and sensitivity to proteases of the host, as the MEP, or by their toxicity in the case of the protein *PorA* and the bacterial porins in general

(Carbonetti, N.H. and Sparling, P.F.; Proc. Natl. Acad. Sci. U.S.A., 84, 9084-9088, 1987). In all the cases the product was obtained as inclusion bodies, as is exemplified for the pTAB9 (FIG. 10C).

In conclusion, it is possible to outline that the use of the stabilizer derivative from the first 47 amino acids of the P64K antigen of *N. meningitidis* (P64K-47) results in an efficiency of expression of heterologous proteins as inclusion bodies, comparable to that of other systems (European patent applications No. 0 416 673 A2 and No. 229 998, Hoechst AG; European patent No. 0 416 673 B1; Castellanos-Sierra, L. R., Hardy, E., Ubieta, R., et al., manuscript submitted), with the additional benefit for the product of being used directly (i.e., without separating it from the stabilizer) due to the absence of meaningful homology with antigens of human origin.

Example 5

The availability of a ligand that recognizes specifically the stabilizer (e.g. an antibody, an enzymatic cofactor, etc.) is a desirable characteristic in any expression system of recombinant proteins. This is due so that the foregoing can permit, for instance, the design of efficient plans of affinity purification if said ligand is immobilize in a chromatographic resin; and even--in the case of the antibodies--the follow-up of the intermediate steps of the purification through immunologic techniques, independently of the identity of the expressed heterologous protein.

MARKED UP VERSION OF THE SPECIFICATION

Such an objective was reached immunizing mice with the protein TAB13 (SEQUENCE IDENTIFICATION NO.: 29 ~~20~~) in order to obtain monoclonal antibodies (MAb) against this stabilizer. TAB13 is an MEP derived from the TAB9 which is different from the former by the presence of two additional V3 consensus regions (FIG. 9B):

C6: TSITIGPGQVFYRTG (SEQUENCE IDENTIFICATION NO.: 24 ~~15~~)

C8: RQRTSIGQGQALYTT (SEQUENCE IDENTIFICATION NO.: 25) ~~16~~

This MEP was expressed (EXAMPLE 4) and purified (EXAMPLE 6) in an analogous way to that described for the TAB4 and TAB9.

Then, mice Balb/c were immunized by subcutaneous route with 3 doses of 20 µg of TAB13 adsorbed to aluminum hydroxide adjuvant at a 15 days-interval. The animal were boosted by intraperitoneal route with 20 µg of the same antigen in buffer phosphate, 20 days after the last dose. The splenocytes were fused with the myeloma X63 Ag8 653 and the resultant hybridomas were isolated and tested according to established methods (Gavilondo, J. V. (ed.), Monoclonal Antibodies: Theory and Practical, Elfos Scientiae, 1995, The Havana, Cuba).

The reactivity of the antibodies secreted by the isolated hybridomas was evaluated by ELISA, coating the plates with the MEP TAB13, the P64K protein or synthetic peptides representing the different V3 regions present in TAB13. In total 18 positive clones were

obtained, one of which, designated 448/30/7, recognized TAB13 as well as 64K, but none of the peptides from the gp120.

The specificity of this MAb by the stabilizer peptide of the pM-83 and the possibility of its use for the immunologic detection of proteins that contain it, was determined by Western blotting, using different samples, heterologous proteins fused to the stabilizer derived from P64K (P64K-47), or the same fused protein or to the first 58 amino acids of the IL-2 (IL2-58). To do this, the *E. coli* strain MM294 was transformed (Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: To Manual Laboratory, 1989, Cold Spring Harbor Laboratory Press, New York, USA) with the following plasmids: pILM-28 (IL2-58+*porA*), pM-82 (P64K-47+*porA*), pTAB13 (P64K-47+MEP), pM-6 (P64K) and pFP15 (IL-2). The expression plasmid pM-134 was also used, which contains the first 120 amino acids of the P64K, which includes the binding domain to the lipoic acid under the control of the same regulatory signals as in the previous plasmids. This segment was amplified by PCR using the primer 1573 (SEQUENCE IDENTIFICATION NO.: 11 ~~2~~) and 2192 (SEQUENCE IDENTIFICATION NO.: 13 4); it was digested with the enzymes NcoI and BamHI, and was cloned in the plasmid pFP15 (see EXAMPLE 4) digested identically. The expression of these transformants was achieved in the growth conditions specified in the EXAMPLE 4 for the pTAB4 and the pTAB9.

The results obtained are represented in FIG. 11. As can be appreciated, MAb 448/30/7 recognizes a probably linear epitope within the stabilizer P64K-47, due to its reactivity with the samples of the plasmids pM-6, pM-82, pTAB13 and pM134 in spite of all these

proteins being antigenically different. This experiment demonstrates that in no case is this reactivity due to the protein fused to the stabilizer (e.g. plasmids pILM-28 and pM-82: both carry the gene *porA* under different stabilizer) which evidences the specificity of recognition of this MAb.

In conclusion, the expression system formed by the stabilizer P64K-47, the plasmids that contain it and MAb 448/30/7 permit the efficient synthesis and in the form of inclusion bodies of a great variety of proteins, and their detection without the previous availability of immunologic probes against each polypeptide to express.

Example 6

The absence of deleterious effects on the immune response against the polypeptide fused to the stabilizer is an important factor to take into account upon selecting an expression system for vaccine candidates. One of the advantages of the expression system based on the stabilizer P64K-47 is precisely its decreased immunogenicity (EXAMPLE 1) which guarantees the foregoing. Nevertheless, the influence of the stabilizer P64K-47 in the immune response against the fused protein was evaluated qualitatively through the comparison of the antibodies response against the different peptides of the V3 region present in the MEP TAB4 (IL2-22) and TAB9 (P64K-47).

For the expression and the purification of TAB4 and TAB9, the biomass of the strain W3110 *trpA905*+pTAB4 and W3110 *trpA905*+pTAB9 was obtained as described in the

MARKED UP VERSION OF THE SPECIFICATION

EXAMPLE 4. This biomass was broken combining the treatment with lyzozyme and with ultrasound in fluoride presence of phenyl methyl sulfonyl (PMSF) and the non-ionic detergent TRITON® X-100 ~~Triton-X-100~~; the inclusion bodies were obtained by differential centrifugation, and the MEP were partially purified and solubilized by two successive wash cycles of the inclusion bodies with chostropic agents and detergents (TAB4: 1. Urea 4 M TRITON® X-100 ~~Triton-X-100~~ 1%, 2. Urea 8 M. TAB9: 1. Urea 8 M TRITON® X-100 ~~Triton-X-100~~ 1%, 2. guanidium chloride 6 M). The supernatants obtained were finally purified through a gradient from 20 to 80% of acetonitrile in a column C4 VYDAC of high performance liquid chromatography (HPLC), being achieved 90% of purity approximately.

The purified recombinant proteins were adsorbed to a gel of aluminum hydroxide adjuvant using a relationship of 60 mg of adjuvant ~~adyuvant~~ per mg of protein. These preparations were used to immunize 5 groups of rabbits by subcutaneous route with 200 µg/dose. The immune response was evaluated by ELISA, using polystyrene plates of 96 wells (High binding, Costar, USA), well coated with the MEP used for the immunization, or with peptides corresponding to each one of the V3 regions present on it. The titers were calculated as the maximum dilution of each serum with an absorbance value of twice higher than that of a mixture of pre immune sera. All the sera were analyzed in duplicate.

The values obtained (Figure 12) show that the titers against the V3 regions are similar between the varying IL2-22 + MEP (TAB4) and P64K-47 + MEP (TAB9). Though the

MARKED UP VERSION OF THE SPECIFICATION

recognition frequency of the peptides is slightly greater for the TAB9, this difference is not meaningful statistically ($p < 0.05$). In conclusion, the immunogenicity of the heterologous protein is affected by the stabilizer P64K-47 in a minimal way, and comparable to other expression systems currently in use.

The hybridoma secreting MAb 448/30/7 was deposited with BCCM™/LMBP, Department of Molecular Biology, Ghent University, Fiers-Schell-Van Montagu Building,Technologiepark 927,B-9052 Zwijnaarde, Belgium, under deposit number LMBP 6047CB.

~~DESCRIPTION OF THE FIGURES~~

~~FIG. 1: Nucleotide sequence of the gene *lpdA* gene coding for P64K. It is shown in *italic* the sequence added in the plasmid pM-6 (European Patent application No. 0 474 313 A2), absent originally in the gene *lpdA*.~~

~~FIG. 2: Reactivity of the polyclonal serum of mouse against peptides of the P64K. A minimal value of 0.4 optical density units to consider the result as positive was chosen.~~

~~FIG. 3: Amino acid sequence of the stabilizer, deduced of the DNA sequence amplified by PCR from plasmid pM-6. The underlined sequences correspond to the oligonucleotide primers.~~

MARKED UP VERSION OF THE SPECIFICATION

~~FIG. 4: Strategy for the construction of plasmid pM-83.~~

~~FIG. 5: Results of the search of homology between the sequences of the stabilizer ('Query') and those present in the SWISS-PROT ('Sbjct') base, using the BLASTP program. The corresponding income for human proteins or for mammals proteins are only shown. P(N) represents the probability of finding N equal alignments within a base composed of random sequences; the significance of the homology diminishes with the value of P(N). Identical residues are represented with their codes of one letter; the conservatives substitutions with a '+', and the differences are not indicated.~~

~~FIG. 6: Results of the search of homology between the sequences of the stabilizer ('Query') and all the possible translations of the sequences of the EMBL Data Library ('Sbjct'), using the program TBLASTN. The corresponding income to human proteins or mammal proteins are only shown. P(N) represents the probability of finding N equal alignments within a base composed of random sequences; the significance of the homology diminishes with the value of P(N). Identical residues are represented with their code of one letter; the conservative substitutions with a '+', and the differences are not indicated.~~

~~FIG. 7: Strategy for the construction of plasmids pTAB4 and pTAB9.~~

~~FIG. 8: Nucleotide and amino acid sequences of the MEP TAB9.~~

MARKED UP VERSION OF THE SPECIFICATION

~~FIG. 9: A: General structure of the MEP TAB4 and TAB9. B: General structure of the MEP TAB13.~~

~~FIG. 10: Comparison of the expression of the genes *porA*, *opc* and the MEP under stabilizer derivatives from the human IL-2 or from the first 47 amino acids of the P64K antigen.~~

~~A: Comparative table. hIL2-58 refers to the first 58 amino acids of the human IL-2, hIL2-22 to the first 22, and P64K-47 to stabilizer derivative from the first 47 amino acids of the P64K antigen.~~

~~B: Comparative analysis by SDS-PAGE of the expression of the MEP in the plasmids TAB4 and TAB9. Lane A: Molecular weight markers; B: Total proteins of the strain W3110 *trpA905*; C: Total proteins of W3110 *trpA905*+pTAB4; D: Purified TAB4; E: Total proteins of W3110 *trpA905* pTAB9; F: Purified TAB9.~~

~~C: Expression of TAB9 in inclusion bodies. A: Soluble proteins of the sample. B: Insoluble proteins or of membrane.~~

~~FIG. 11: Western blotting using MAb 448/30/7 with total protein samples of *E. coli* MM294 transformed with: 1: Negative control, 2: pM-6 (P64K), 3: pM-82 (P64K-47+*porA*), 4: pTAB13 (P64K-47+MEP), 5: pFP15 (IL-2), 6: pM-134 (P64K-120), 7: pILM-28 (IL2-58+*porA*). The molecular weight markers are indicated on the left.~~

MARKED UP VERSION OF THE SPECIFICATION

~~FIG. 12: Reciprocal of the titer value by ELISA of the rabbits immunized with TAB4 and TAB9. GM: Geometric mean of the reciprocal of the titers anti V3; R: Percent of reactivity with the V3 peptides.~~

MARKED UP VERSION OF THE ABSTRACT

ABSTRACT

The present invention related to biotechnology and genetic engineering, particularly the expression of proteins of viral origin in microorganisms through their fusion~~,~~ by applying ~~the~~ recombinant DNA technology~~,~~ to bacterial peptides. The present invention provides an efficient process for the expression in *Escherichia coli* of heterlogous proteins as fusion peptides with a view to obtaining them with a high degree of purity, in commercially useful amounts, and in an appropriate form for their inclusion in vaccine preparations ~~intended to human use~~. ~~To this effect, what~~ What is essentially used is a stabilizing sequence derived from the first 47 amino acids of the antigen P64k of *Neisseria meningitides* B:4:P1.15. In particular, use is made of a recombinant plasmid containing said sequence, under the control of the tryptophane promoter of *E. coli* and of the terminator of the transcription of the phage T4, including restrictions sites which provide for the cloning in phase of DNA fragments coding for polypeptides of interest. ~~The process of the invention is applicable to the pharmaceutical industry, for the development of diagnostic systems, vaccine preparations, and in any situation where it is required to obtain high amounts of heterologous proteins as fusion polypeptides in *E. coli*.~~

B-cell epitope mapping of the *Neisseria meningitidis* P64k protein using overlapping peptides

Sonia González*[1], Lisel Viña*[2], Consuelo Nazábal*, Glay Chinea†, Evelin Caballero* and Alexis Musacchio*

**Division of Vaccines, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana 10600, Cuba, and †Division of Physical-Chemistry, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana 10600, Cuba*

A common meningococcal antigen designated P64k has been identified, cloned and expressed in *Escherichia coli*. The recombinant antigen is highly immunogenic in several animal species and its immunogenicity in healthy human volunteers is under investigation. Recently, P64k has been used as an immunological carrier for weak immunogens. To characterize the B-cell epitopes on P64k, recognized by immune sera obtained from mice, rabbits and monkeys, multiple overlapping peptides were synthesized and screened for antibody binding. Peptides covering the complete sequence of the P64k protein, 59 in all, of 20 amino acids each (overlapped by 10 residues), were synthesized. A number of continuous epitopes were detected with all sera, when immune and pre-immune bleeds were compared. For mouse and monkey sera, a few major antigenic peptides were identified, while the recognition of the rabbit serum was much more heterogeneous. Despite variation in the exact location of continuous epitopes defined by different anti-P64k sera, we found an immunogenic core region within the molecule, composed of amino acids Asp^{524}–Gly^{533}. Consistently, in this protein segment there was an amino acid stretch located in a β-hairpin loop, which is exposed to the solvent in the previously determined three-dimensional structure of the protein. This region is protruding and accessible to a sphere with a radius of 9 Å.

Introduction

Neisseria meningitidis is a pathogen responsible for a serious invasive disease throughout the world [1]. The lack of an effective vaccine against all serogroups constitutes a problem in the control of meningococcal disease [2]. Several outer-membrane proteins (OMPs) have been examined as vaccine candidates [3]. Besides major OMPs, other surface proteins are under investigation in several laboratories, including highly conserved OMPs that could potentially confer protection against meningococcal disease in humans [4].

Our group has previously isolated, cloned and expressed in *Escherichia coli* the gene *lpdA*, encoding a high-molecular-mass protein (P64k) that is common to many meningococcal isolates [5]. To further characterize this antigen, we have evaluated its immunogenicity in mice, rabbits and monkeys. The antigen was highly immunogenic in all three animal species [6], inducing antibodies that recognized the natural antigen in the membrane of meningococci. Besides, we have generated a group of monoclonal antibodies (mAbs) against the recombinant protein, which recognize four non-overlapping epitopes, as shown using competition assays with biotinylated mAbs [7]. This recognition was further confirmed by epitope mapping of the protein using the Geysen (PEPSCAN) procedure [7a].

Studying the sequence of the protein, it was found that it has a high degree of homology with two enzymes of the pyruvate dehydrogenase complex of several species. The first 110 amino acids are similar to those in the lipoyl-binding domain present in the enzyme dihydrolipoamide acetyltransferase (E2). The rest of the protein has homology with the enzyme lipoamide dehydrogenase (E3) [8]. This enzyme contains a flavin adenine dinucleotide (FAD)-binding domain near the N-terminal end.

Similarly, there is a FAD molecule associated with the recombinant P64k protein obtained from *E. coli* (holoenzyme). Moreover, its lipoamide dehydrogenase activity *in vitro* has been determined [9]. The FAD molecule can be separated from its binding site when the protein is denatured, yielding the apoenzymic form of P64k.

Recently, this recombinant protein has been used as an immunological carrier for peptide antigens in experimental animals [10], as well as in human volunteers [11], recipient of an anti-cancer vaccine candidate. The protein has also been employed as a pilot protein for the insertion of selected epitopes of foreign antigens [12,13].

With the intention of identifying the major antigenic regions in the protein, we decided to scan multiple over-

Key words: meningococci, P64k protein, synthetic peptide.

Abbreviations used: mAb, monoclonal antibody; 3-D, three-dimensional; ABTS, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid); HRP, horseradish peroxidase; H P64k, holoenzyme form of P64k; A P64k, apoenzyme form of P64k.

[1] To whom correspondence should be addressed (e-mail sonia.gonzalez@cigb.edu.cu).

[2] Current address: Center of Molecular Immunology, P.O. Box 16040, Havana 11600, Cuba

lapping peptides, corresponding to the entire sequence of the recombinant P64k molecule, with antisera obtained in three animal species. The core immunogenic region mapped by these sera was allocated in the three-dimensional (3-D) structure of this antigen, previously determined by X-ray crystallography [14,15].

Materials and methods

Animals

Female Balb/c mice (6–8 weeks) and New Zealand White rabbits were from the National Center for Laboratory Animals (Havana, Cuba). Male *Cercopitecus aethiops* monkeys were supplied by the same institution. All the experimental procedures performed in animals were approved by the Institutional Ethic Committee of the Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba.

Reagents

Recombinant P64k protein was obtained as described earlier [16]. The final purity was higher than 99%, as ascertained by SDS/PAGE [17], immunoblotting [18] with mAbs and by ELISA [19] specific to the host contaminants. The protein
was obtained in the holoenzyme form (H P64k). Its apoenzyme form (A P64k) was prepared by precipitating pure holoenzyme with 10% trichloroacetic acid. The pellet fraction was suspended in 0.1% NaOH and the suspension was dialysed against PBS. The protein content of both enzyme forms was determined by the Lowry method [20].

BSA, complete and incomplete Freund's adjuvant and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were all from Sigma (St. Louis, MO, U.S.A.). Aluminium hydroxide was purchased from Superfos Biosector, Vedbaek, Denmark. The rest of the chemicals were all purchased from Merck, Darmstadt, Germany.

Microplates for epitope scanning were from Chiron Technologies, Clayton, Victoria, Australia. In the last step Costar-Medium Binding plates (Corning Costar, Cambridge, MA, U.S.A.) were used. Goat anti-mouse Ig conjugated to horseradish peroxidase (HRP), anti-rabbit Ig conjugated to HRP and anti-human Ig conjugated to HRP were all from Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.

Generation of polyclonal anti-P64k sera

Two rabbits were immunized subcutaneously with 100 μg of H P64k diluted in PBS and emulsified in an equal volume of complete Freund's adjuvant. On days 14 and 28 the rabbits were boosted with the same amount of protein with incomplete Freund's adjuvant. Bleedings were performed on days 0 and 35 of the immunization schedule. Six mice per group were injected subcutaneously with 20 μg of either H P64k or A P64k, absorbed on 0.8 mg of aluminium hydroxide. Similar booster immunizations were given on days 14 and 28. On days 0 and 35 the mice were bled for collection of the pre-immune and post-immune sera, respectively. A third group of mice received 20 μg of H P64k emulsified in Freund's adjuvant, as described above for rabbit antisera production. Two monkeys were immunized intramuscularly with 50 μg of A P64k, absorbed on 2 mg of aluminium hydroxide. Equal booster doses were given by the same route on days 30 and 60. The monkeys were bled for collection of sera on days 0 and 75.

Sera obtained in the three species were evaluated by ELISA on plates coated with H P64k (1 μg/ml) as described earlier [21]. Species-specific anti-Ig antibodies conjugated to HRP were employed as second antibodies in the immunoassay, to detect mouse and rabbit anti-P64k antibodies. Anti-human Ig antibody conjugated to HRP was employed to detect the immune response in the monkey sera.

Multiple solid-phase synthesis

Peptides of 20 amino acids with a 10-residues overlap corresponding to the amino acid sequence of P64k were assembled on to activated polyethylene pins. On one block of pins were accommodated 59 peptides.

The overlapping peptide panel was synthesized using the Multipin non-cleavable peptide kit (Chiron Technologies), adhering strictly to the protocol recommended by the manufacturer. After completion of synthesis, all peptides were acetylated routinely at the N-terminus with a 50:5:1 (by vol.) mixture of dimethylformamide, acetic anhydride and triethylamine. Side-chain deprotection was accomplished over a 2-h period at room temperature with a 38:1:1 (by vol.) mixture of trifluoroacetic acid, ethanedithiol and anisole.

Epitope mapping of P64k

Analysis of the continuous epitopes recognized by polyclonal antibodies against P64k was performed using multiple solid-phase peptides according to the manufacturer's instructions. Briefly, pins were blocked by addition of 0.1% (v/v) Tween 20 in PBS, containing 2% BSA (200 μl/well). After 1 h, the pins were washed for 10 min in PBS. They were then transferred to wells containing 175 μl/well of the antibodies diluted in blocking buffer, and incubated overnight at 4 °C. The serum dilution for mouse, rabbit and monkey 2 sera was 1:1000, and for monkey 1 serum was 1:300. The block of pins was washed four times with PBS, 10 min per wash. Afterwards, the block was incubated with 175 μl/well of suitable (γ-chain-specific) HRP conjugate diluted in PBS containing 0.1% (w/v) BSA, 0.1% (v/v) normal goat serum and 0.1% (v/v) Tween 20. Following incubation for 1 h at room temperature, pins were washed four times in PBS (as

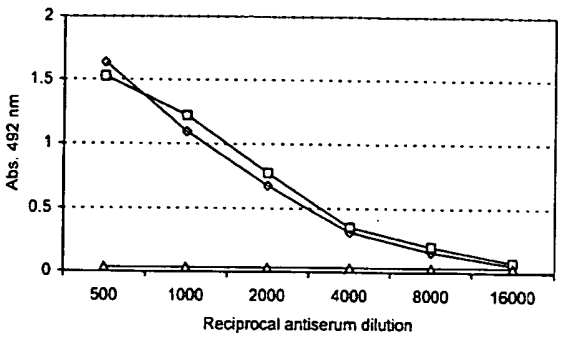


Figure 1 Reactivities of monkey anti-P64k sera as determined by ELISA

Titres are indicated for monkey antiserum 1 (□), monkey antiserum 2 (◇) and pre-immune monkey serum (△).

before). Then, pins were placed in 175 μl/well of substrate buffer (0.1 M Na_2HPO_4/0.1 M citric acid, pH 4.0) containing 1 mg/ml ABTS and 0.01% hydrogen peroxide. Colour development was monitored over 10–45 min. To reduce the possibility of a false negative, we made all readings dual wavelength (405–492 nm). All antibody-incubation and washing steps were performed on a flat-bed table shaker at 100 rev./min.

After each screening, the antibodies were removed from pins by sonication in disruption buffer containing 1% (w/v) SDS and 0.1% (v/v) 2-mercaptoethanol in PBS, at 60 °C for 10 min. Then, pins were washed in distilled water at an initial temperature of 65 °C for 30 min with agitation. Finally, they were immersed in methanol at 60 °C for 65 s, followed by air-drying. Occasionally, the efficient removal of antibody from pins was confirmed by testing them with the appropriate conjugate. For each antibody sample, at least two epitope scans were performed.

Location of epitopes in the 3-D structure of P64k

A three-dimensional model of the P64k's domain Gly^{117}–Lys^{598}, obtained by X-ray crystallography [15], was used in the analysis. Visual inspection of the model and

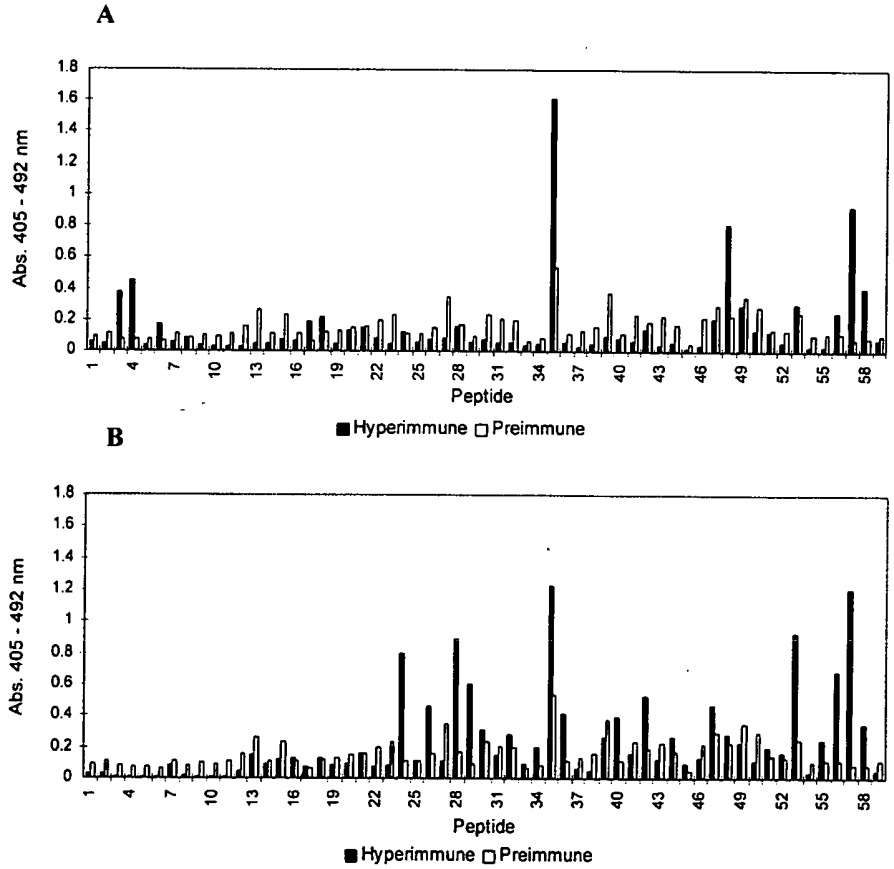


Figure 2 Scanning-epitope analysis of murine polyclonal anti-P64k sera

Indicated are the reactivities of pooled sera directed to either H P64k (A) or A P64k (B). The numbers in the abscissa indicate 20-amino acid peptides with 10-residues overlap corresponding to the amino acid sequence of P64k. The absorbance values represent the averages of two independent scanning experiments, where the background values were 0.029 and 0.018, respectively.

determination of surface accessibility and secondary structure was done using WHATIF [22]. Protrusion was calculated by the method reported by Thornton and co-workers [23].

Results

Polyclonal anti-P64k antibodies

As expected, experimental animals seroconverted in response to the immunization with P64k protein. All hyperimmune sera were tested by ELISA. The specific immune response obtained in monkeys is represented in Figure 1. The antibody titres for mice and rabbits were higher than those obtained in monkeys (results not shown).

Epitope specificity of antibodies in PEPSCAN

We analysed the continuous epitopes recognized by immune sera obtained in three animal species. Individual rabbit and monkey sera (pre- and post-immunization) sera were assayed. The murine antisera were pooled before they were tested. The mean of the 12 lowest absorbance readings (corresponding to the bottom 20% of readings) was taken as

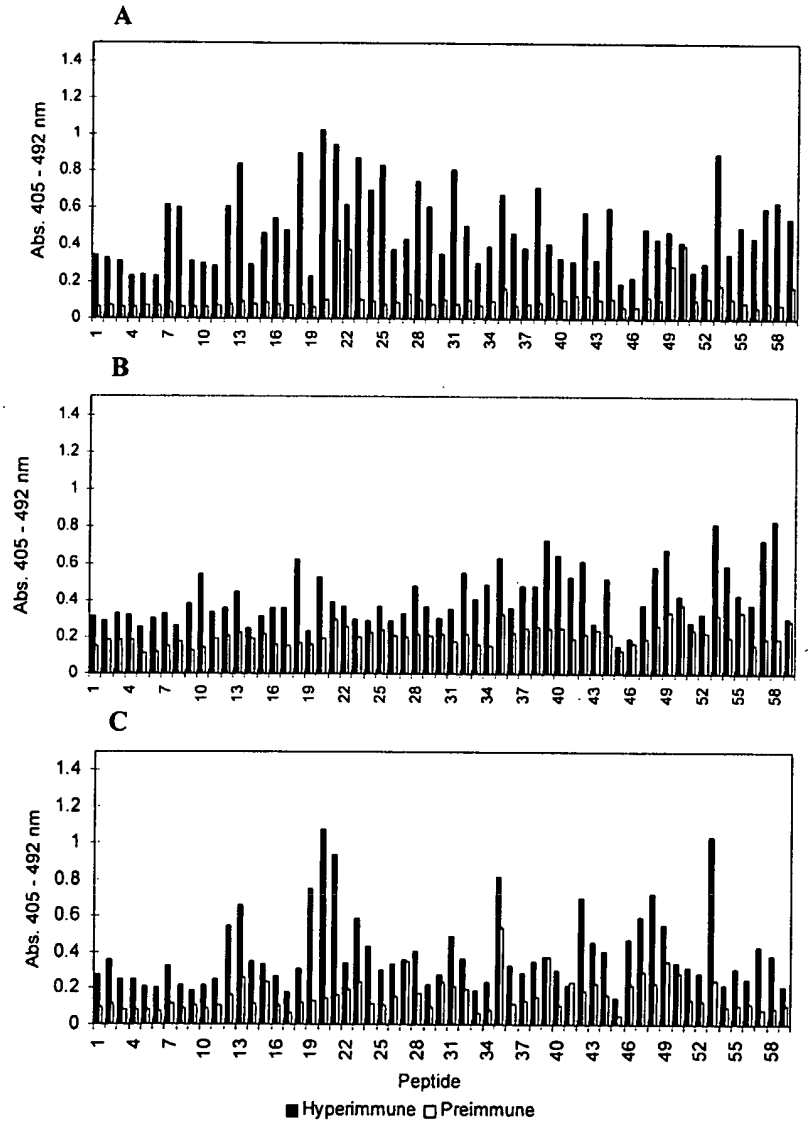


Figure 3 Scanning-epitope analysis of rabbit and murine polyclonal anti-H P64k sera

Indicated are the profiles obtained for rabbit antiserum 1 (A), rabbit antiserum 2 (B) and mouse pooled sera (C). The numbers in the abscissa indicate 20-amino acid peptides with 10-residues overlap corresponding to the amino acid sequence of P64k. The absorbance values represent the averages of two independent scanning experiments, where the background values were 0.258, 0.25 and 0.203, respectively.

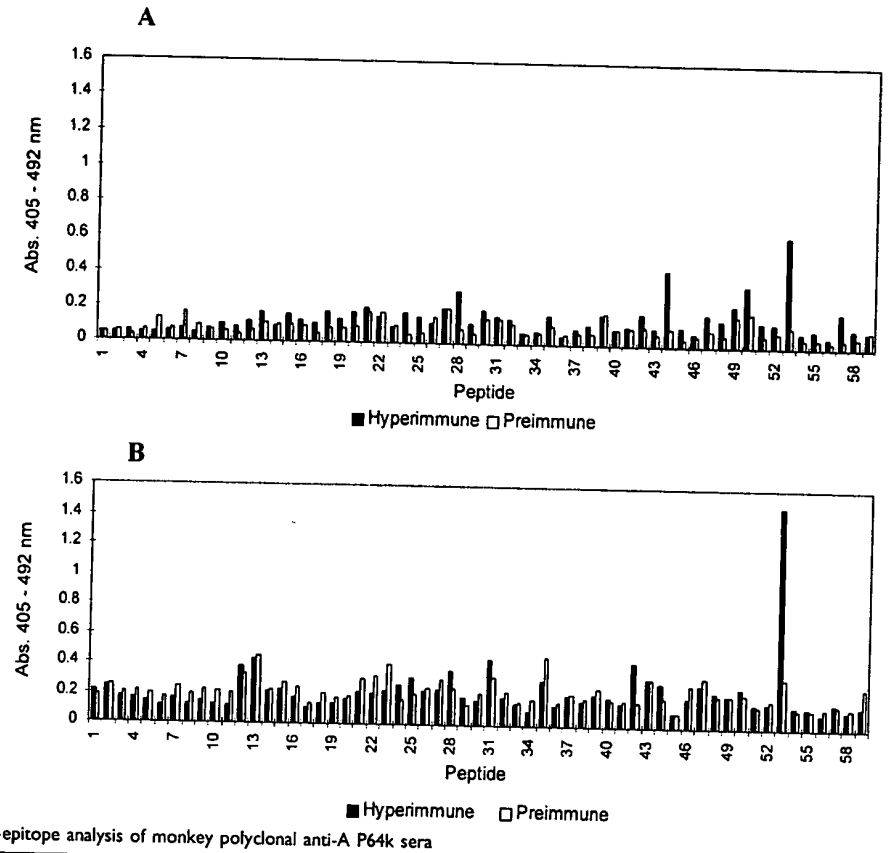


Figure 4 Scanning-epitope analysis of monkey polyclonal anti-A P64k sera

Indicated are the profiles obtained for monkey antiserum 1 (A) and monkey antiserum 2 (B). The numbers in the abscissa indicate 20-amino acid peptides with 10-residues overlap corresponding to the amino acid sequence of P64k. The absorbance values represent the averages of two independent scanning experiments, where the background values were 0.057 and 0.116, respectively. The reactivity of mouse pooled sera, obtained with similar conformation of protein and adjuvant, can be observed in Figure 2(B).

a cut-off value for each positive serum, as published elsewhere [7a].

Figure 2 shows the reactivity of murine polyclonal antibody obtained against both statuses of the recombinant protein, the apoenzyme and the holoenzyme, with a panel of overlapping peptides comprising the amino acid sequence of the protein. In both cases aluminium hydroxide was employed as an adjuvant. As seen in Figure 2(A), when the holoenzyme form of the protein was employed for the immunization there were three major antigenic sites in peptides 35, 48 and 57. Pooled sera of mice injected with the apoenzyme form of the recombinant protein reacted with five peptides (peptides 24, 28, 35, 53 and 57), as shown in Figure 2(B). Antisera prepared against both protein conformations recognized peptides 35 and 57, suggesting two major antigenic cores of the antigen for this species.

In Figure 3, the reactivity of overlapping peptides with antisera obtained against H P64k in two species can be observed. Compared with murine pooled sera, the two rabbit antisera reacted with a broader range of overlapping peptides, giving a relatively unspecific response. Figure 3(A) shows that the highest signals (absorbance of the hyperimmune serum/absorbance of the preimmune serum) for rabbit immune serum 1 were obtained with peptides 13, 18, 20, 23, 25, 31 and 53. Rabbit antiserum 2 reacted better with peptides 39, 53, 57 and 58 (Figure 3B). Then, differences in the peptides recognized by the two rabbit sera could be seen, in spite of the heterogeneity of the response in these two animals. Mouse pooled sera gave the highest absorbance values with peptides 19, 20, 21 and 53 (Figure 3C).

It can be observed in Figure 4 that monkey anti-A P64k sera recognized mainly an epitope present in peptide 53, having very low reactivity with the rest of the peptides. One of these sera reacted with a second peptide (peptide 44), but with lower intensity (Figure 4A). Taking into account the epitope scan shown in Figure 2(B), which corresponds to mouse pooled sera obtained under the same conditions, it is evident that divergences in the reactivity pattern among different species are bigger than those among individual sera of the same species.

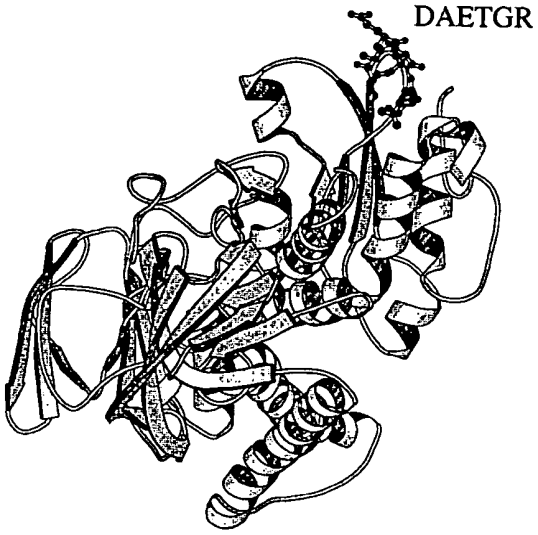


Figure 5 Location in the 3-D structure of P64k of the immunogenic core region recognized by various anti-P64k sera

The structure was experimentally determined by Li and co-workers [15]. The Figure was prepared using the MOLSCRIPT program of Per Kraulis [38].

Despite variation in the exact location of continuous epitopes, defined by different anti-P64k, we found that the C-terminal portion of the protein was more antigenic than the N-terminal one, in terms of more continuous epitopes recognized by different antisera. Additionally, inside the C-terminal portion of the protein, we found that peptide 53 (TKLIFDAETGRIIGGGIVGP) was recognized by most of the sera analysed in this study. Taking into account that the sera employed in this study did not recognize the overlapping peptides 52 and 54, we assigned the marked antigenicity to the central portion of peptide 53, which sequence is DAETGRIIGG.

Location of the immunogenic core region in the 3-D structure of P64k

When we looked at the 3-D structure of P64k, experimentally determined by X-ray crystallography, we found that the residues DAETGR, in the core region of peptide 53, are located in a β-hairpin loop and exposed to the solvent. This region is protruding, accessible to a sphere of 9-Å radius (Figure 5).

Discussion

Murine pooled sera obtained against the two enzymic forms of recombinant P64k protein were analysed by PEPSCAN mapping. When we compared the obtained epitope specificity, we found that the holoenzymic form of P64k induces slightly lower amounts of antibodies against linear epitopes than its apoenzymic form (Figure 2). It could be due to differences in their thermostability, which have been reported for other lipoamide dehydrogenases [25,26].

When studying the main immunogenic regions recognized by rabbit sera, we found the same broader reactivity that other researchers have observed for this species [27,28], compared with the recognition given by mouse pooled sera obtained by the same procedure. All sera whose reactivities are shown in Figure 3 were obtained by immunizing animals with the antigen emulsified with Freund's adjuvant. In a previous study [29], we compared the specificity of murine anti-P64k sera directed to the antigen adjuvanted with either Freund's adjuvant or aluminum hydroxide. The solid-phase antigen used in both studies was the described panel of overlapping peptides, and a broader reactivity for the murine pooled sera obtained with Freund's adjuvant was observed. It might suggest that Freund's adjuvant favoured the production of antibodies that better react with epitopes present in the unfolded protein. Kenney and co-workers [30], who found that Freund's adjuvant produced antibodies reactive to denatured protein, also suggested this fact. Additionally, it has been speculated that the emulsification step leads to the unfolding of the antigen, giving place to a preferential recognition of linear epitopes by the generated antibodies [31]. Moreover, ten Hagen and co-workers [32] have suggested that the antigen emulsified in water-in-oil emulsions is only able to interact with the cell surface once the oil-emulsion droplets have been ingested and digested within cells, especially macrophages. Therefore, the antigen is presented in fragments after degradation, which might reduce the number the conformational antigenic determinants. Taking together the broad reactivity found for rabbit sera and the adjuvant employed to obtain these anti-P64k sera, our results could be explained.

Monkey antisera recognized very few overlapping epitopes, mainly peptide 53. Several researchers have found very low, if any, reactivity of human antibodies with linear peptides [33,34], suggesting that the human response is preferentially formed by antibodies directed against discontinuous epitopes. Similar behaviour for monkey antibodies could explain the low reactivity with overlapping peptides found in this study. Another report suggests that a high background can be expected from human antibodies (and to a lesser extent for monkey sera), due to a broad sensitization of humans [7a]. Our results match better with the former observations.

Alteration in the conformation of the antigen could produce different specificity in the B-cell response. The reactivity pattern observed for mouse sera in this study supports the influence of this factor, which has also been found to be important by Vordermeier and co-workers [35].

Antibodies produced in different species of experimental animals, obtained under the same conditions, reacted with distinct overlapping peptides in the same antigen. Others [27,28] have reported the same result, which was expected due to the influence of genetic factors [36].

In this study, we found a higher B-cell response to the C-terminal end of the protein. This is in agreement with the epitopes recognized by several murine mAbs directed against this protein, which have been mapped by the same method (C. Nazábal, S. Cruz, T. Carmenate, A. Musacchio, R. Silva, M. Delgado, S. González and G. Chinea, unpublished work). However, it has been previously observed in our group, when raising mAbs against hybrid proteins containing the first amino acids of P64k, that the N-terminal end is highly immunogenic in mice [37]. It could be explained as a different immunogenicity and antigenicity displayed by this segment of the molecule. It is also possible that the N-terminal portion of the protein only becomes immunodominant when inserted in another molecule, but not when it is in the original protein, where the rest of the protein, mainly the C-terminal portion, seems to be more immunogenic and/or antigenic.

B-cell epitopes in native proteins generally are hydrophilic amino acids on the protein surfaces that are topographically accessible to free antibodies [36]. In spite of observed differences in the recognition of the P64k overlapping peptides by different antisera, we found a common antigenic region in peptide 53. Consistently, the sequence DAETGR, located in an exposed loop of the protein, is accessible to the antibodies, as can be seen in from the 3-D structure of the protein (Figure 5).

P64k protein has been evaluated as a carrier for peptide and polysaccharide antigens. The characterization of the B-cell response elicited by the protein is an important factor for the identification of candidate regions in it, which could be used as a frame to express foreign epitopes in hybrid vaccine candidates. The present study is a contribution to such an approach. Mapping of B-cell continuous epitopes recognized by human antibodies directed against this protein is in progress and it will certainly improve our knowledge of this bacterial antigen.

Acknowledgments

We are grateful to Dr. Kanury Rao, from the International Center for Genetic Engineering and Biotechnology (ICGEB), New Delhi Component, for his support to C.N. in the design and synthesis of overlapping peptides. This work was partially supported by a Collaborative Research Program, Project CRP/CUB 94-02, from ICGEB, Trieste, Italy.

References

1 Achtman, M. (1995) in Meningococcal Disease (Cartwright, K., ed.), pp. 159–175, John Wiley and Sons, Chichester
2 Poolman, J. T. (1995) Infect. Agents Dis. **4**, 13–28
3 Saukkonen, K., Leinonen, M., Abdillahi, H. and Poolman, J. T. (1989) Vaccine **7**, 325–328
4 Frasch, C. E. (1989) Clin. Microbiol. Rev. **2** (suppl.), S134–S138
5 Silva, R., Menéndez, T., Alonso, L. M., Iglesias, E., Musacchio, A., Leal, M. J., Alvarez, A., Coizeau, E., Martín, A., Herrera, L. et al. (1999) FEMS Microbiol. Lett. **174**, 191–199
6 Guillén, G., Silva, R., Alvarez, A., Coizeau, E., Novoa, L. I., Selman, M., Morales, J., González, S., Musacchio, A., del Valle, J. et al. (1994) in Pathobiology and Immunobiology of *Neisseriaceae* (Conde-Glez, C. J., Morse, S., Rice, P., Sparling, F. and Calderón, E., eds.), pp. 834–840, Instituto Nacional de Salud Pública, Cuernavaca
7 Nazábal, C., Cruz, S., Carmenate, T., Musacchio, A., Silva, R., Delgado, M., González, S., Guillén, G. and Herrera, L. (1994) Proceedings of the Ninth International Pathogenic Neisseria Conference, Winchester, U.K. (Evans, J. S., Yost, S. E., Maiden, M. C. J. and Feavers, I. M., eds.), p. 98, S. C. C., Reading
7a Carter, J. M. (1994) Methods Mol. Biol. **36**, 207–223
8 Bringas, R. and Fernández, J. R. (1995) Protein Struct. Funct. Genet. **21**, 303–306
9 Gómez, R., Madrazo, J., González, J., Chinea, G., Musacchio, A., Rodríguez, A. and Padrón, G. (1999) Biotecnol. Apl. **16**, 83–87
10 Pardo, O. L., González, S., García, J. L., Viña, L. and Guillén, G. (1998) Proceedings of the Eleventh International Pathogenic Neisseria Conference, Nice, France (Nassif, X., Quentin-Millet, M. J. and Taha, M. K., eds.), p. 192, Editions E.D.K., Paris
11 González, G., Crombet, T., Catalá, M., Mirabal, V., Hernández, J. C., González, Y., Marinello, P., Guillén, G. and Lage, A. (1998) Ann. Oncol. **9**, 1–5
12 Guillén, G., Alvarez, A., Niebla, O., Silva, R., González, S., Musacchio, A., Martín, A., Delgado, M. and Herrera, L. S. (1996) Acta Biotechnol. **16**, 165–173
13 Guillén, G., Alvarez, A., González, S., Musacchio, A., Niebla, O., Coizeau, E., Silva, R., Martín, A. and Herrera, L. (1996) Biotecnol. Apl. **13**, 271–275
14 Li, I., Prangé, T., Fourmé, R., Padrón, G., Fuentes, P., Musacchio, A. and Madrazo, J. (1994) J. Mol. Biol. **235**, 1154–1155
15 Li, I., Pernot, L., Prangé, T., Saludjian, P., Schiltz, M., Fourmé, R. and Padrón, G. (1997) J. Mol. Biol. **269**, 129–141
16 Guillén, G., Alvarez, A., Silva, R., Morera, V., González, S., Musacchio, A., Besada, V., Coizeau, E., Caballero, E., Nazábal, C. et al. (1998) Biotechnol. Appl. Biochem. **27**, 189–196
17 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
18 Towbin, H. and Gordon, J. (1984) J. Immunol. Methods **72**, 313–340
19 Anicetti, V. R., Fehskens, E. F., Reed, B. R., Chen, A. B., Moore,

P., Geier, M. D. and Jones, A. S. (1986) J. Immunol. Methods **91**, 213–224
20 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
21 Expósito, N., Mestre, M., Silva, R., Nazábal, C., Peña, M., Martínez, N., Font, M. and Guillén, G. (1999) Biotechnol. Appl. Biochem. **29**, 113–119
22 Vriend, G. (1990) J. Mol. Graphics **8**, 52–56
23 Thornton, J. M., Edwards, M. S., Taylor, W. R. and Barlow, D. J. (1986) EMBO J. **5**, 409–413
24 Reference deleted
25 van Berkel, W. J. H., Benen, J. A. E. and Snoek, M. C. (1991) Eur. J. Biochem. **197**, 769–779
26 Mattevi, A., Obmolova, G., Kalk, K. H., van Berkel, W. J. H. and Hol, W. G. J. (1993) J. Mol. Biol. **230**, 1200–1215
27 Das, M. and Lindstrom, J. (1991) Biochemistry **30**, 2470–2477
28 Beattie, J., Fawcett, H. A. C. and Flint, D. J. (1992) Eur. J. Biochem. **210**, 59–66
29 González, S., Nazábal, C., Viña, L. and Caballero, E. (1998) Dev. Biol. Stand. **92**, 269–276
30 Kenney, J. S., Hughes, B. W., Masada, M. P. and Allison, A. C. (1989) J. Immunol. Methods **121**, 157–166
31 Steward, D. (1994) The Theory and Practical Application of Adjuvants, John Wiley and Sons, Chichester
32 ten Hagen, T. L. M., Sulzer, A. J., Kidd, M. R., Lal, A. A. and Hunter, R. L. (1993) J. Immunol. **151**, 7077–7083
33 Meeker, H. C., Williams, D. L., Anderson, D. C., Gillis, T. P., Schuller-Levis, G. and Levis, W. R. (1989) Infect. Immun. **57**, 3689–3694
34 Rouppe van der Voort, E. M., Kuipers, B., Brugghe, H. F., van Unen, L. M. A., Timmermans, H. A. M., Hoogerhout, P. and Poolman, J. T. (1997) FEMS Immunol. Med. Microbiol. **17**, 139–148
35 Vordermeier, H. M., Harris, D. P., Moreno, C., Singh, M. and Ivanyi, J. (1995) Int. Immunol. **7**, 559–566
36 Kuby, J. (1997) in Immunology (Allen, D., Russel, M., Cimino, D. and Steyn, R., eds.), pp. 87–106, W. H. Freeman and Company, New York
37 Guillén, G., Duarte, C., Alvarez, A., Carpio, E., Quintana, D., Gómez, C. E., Silva, R., Nazábal, C., Leal, M. J. and Martín, A. (1997) PCT Patent Application WO 97/26359
38 Kraulis, P. J. (1991) J. Appl. Crystallogr. **4**, 946–950

Received 15 December 1999; accepted 2 March 2000